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Arthrinium rasikravindrii sp. nov. from Svalbard, Norway

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Abstract — *Arthrinium rasikravindrii* sp. nov. was isolated from soil collected in the arctic archipelago Svalbard, Norway. The new species, distinguished by morphological and in vitro cultural characters, forms dark brown lenticular conidia with hyaline equatorial germ slits together with balloon-shaped, anomalous conidia uncommon in the morphologically similar *A. phaeospermum*. Additionally, internal transcribed spacer (ITS) rDNA sequence analyses support this species as distinct within *Arthrinium*. Isolates previously identified as *A. phaeospermum* from China and Japan are re-determined as *A. rasikravindrii*.

Key words — anamorphic fungi, Ny-Ålesund, phylogeny, taxonomy

Introduction

The study of fungal diversity in soils from Svalbard, Norway (Elvebakk et al. 1996, Aarnæs 2002, Kurek et al. 2007), has led to the discovery of novel genera and species from this region (Pang et al. 2008, 2009). During research of the soil mycobiota of this Arctic region, a previously unknown *Arthrinium* was isolated from a soil sample. *Arthrinium* Kunze was established in 1817 with *A. caricicola* Kunze as the type species (Kunze 1817). The genus currently comprises about 31 species worldwide (Kirk et al. 2008), of which 13 have been reported from Svalbard (Aarnæs 2002). Most *Arthrinium* species produce dark, non-septate, mostly lenticular conidia with a hyaline rim or germ slit. Since Ellis (1965, 1971, 1976) extensively treated the genus, several species have been added (Calvo & Guarro 1980, Larrondo & Calvo 1990, 1992). This paper describes and illustrates another new *Arthrinium* species isolated from arctic soil near Svalbard, Norway.

Materials & methods

Sampling site

The soil sample was collected from Ny-Ålesund (78°55′N 11°56′E) on the west coast of Spitsbergen, the largest island in the Svalbard archipelago. Topographically, Ny-Ålesund includes east and west glaciers, terminal moraines, and glacial streams and rivers flowing towards Kongsfjord. The sampling site was situated near an Austre Brøggerbeen glacier (78°55.082′N 11°51.527′E). Mean temperatures are –14°C in the coldest month (February) and 5°C in the warmest month (July–August) (Nygaard 2009). The loose soil texture supports a tundra vegetation (Klimowicz & Uziak 1998).

Isolation, pure culture, and microscopic examination

The soil was sampled from the surface to 5 cm depth from the Ny-Ålesund region during the first (2007) Indian Arctic expedition. The samples were placed in sterile ampoules (Hi-media) and stored at –20°C until studied. For the isolation of fungi, 1 gm of soil sample was taken and serially diluted up to 10^{-7} (Waksman 1916). A soil suspension (1 ml) was used as inoculum on four different culture media — Martin's Rose Bengal medium (MRB), Malt Extract Agar (MEA), Corn Meal Agar (CMA), and Potato Dextrose Agar (PDA). The plates were incubated at 15°C for 10 days. Colonies showing different morphological features were purified and transferred onto PDA slants for further study. Sporulating cultures were identified based on morphology using standard literature (Ellis 1971, 1976, Carmichael et al. 1980, Domsch et al. 1980, Larrondo & Calvo 1990, 1992). We recorded and photographed microscopic details from specimens mounted in lactophenol-cotton blue and distilled water using an Olympus CX-41 microscope. Fungal structures were measured with an ocular micrometer. A pure culture is deposited in the National Fungal Culture Collection of India (NFCCI-WDCM 932), MACS' Agharkar Research Institute, Pune, India.

Polymerase chain reaction (PCR) and sequencing

Total DNA was extracted from a culture grown on a PDA plate for two weeks at 15°C by the high salt DNA extraction method of Aljanbi & Martinez (1997) using Fast Prep-24 tissue homogenizer (MP Biomedicals GmbH, Germany). ITS fragments were amplified using primer pairs ITS4 and ITS5 (White et al. 1990). PCR was performed in a 25 µl reaction using 2 µl template DNA (10–20 ng), 0.5 U Taq DNA polymerase (Genei, Bangalore, India), 2.5 µl 10X Taq DNA polymerase buffer, 0.5 µl 200 µM of each dNTP (Genei, Bangalore, India), 0.5 µl 10 pmol primer, H_2O (Sterile Ultra Pure Water, Sigma) qsp 25 µl. Amplification was performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following parameters: 5 min step at 95°C, followed by 30 cycles of 1 min at 95°C, 30s at 56°C, and 1 min at 72°C for ITS region amplification, then a final 7 min extension step at 72°C. The PCR products were purified with Axygen PCR cleanup kit (Axygen Scientific Inc, CA, USA) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The cycle sequencing products were run on an ABI Avant 3100 automated DNA sequencer (Applied Biosystems, USA).

Sequence alignment and phylogenetic analysis

Sequence alignment of internal transcribed spacer and 5.8S regions of *Arthrinium* sp. (NFCCI 2144) was performed using the Clustal W option of the software Molecular

Table 1. Percent ITS sequence similarity of *Arthrinium rasikravindrii* with Genbank sequences from related *Arthrinium* species.

Species	Culture #	Genbank #	Positions with base changes	Total aligned sequence bp	Sim. w NFCCI2144 (%)*
A. rasikravindrii	NFCCI 2144	JF326454	-	-	-
#	IFO 6575	AB220266	1	501	99.8
#	MAFF 305708	AB220272	1	501	99.8
#	DLEN2008007	GU266274.1	1	501	99.8
#	L 10-2-2	HM008625.1	3	501	99.4
#	L 3-4-2	HM008624.1	2	501	99.6
#	MAFF 410785	AB220273.1	3	501	99.4
A. aureum Calvo & Guarro	CBS 244.83	AB220251	12	502	97.6
	IMI 252326D	AB220246	12	502	97.6
A. arundinis (Corda) Dyko & B. Sutton	IMI 285638B	GU566268	86	521	83.5
A. euphorbiae M.B. Ellis	G41	GU566268	67	503	86.7
A. hispanicum Larrondo & Calvo	IMI 326877	AB220242	64	521	87.7
A. marii Larrondo & Calvo	CBS 497.90	AB220252	65	522	87.5
A. mediterranei Larrondo & Calvo	IMI 326875	AB220243	62	521	88.0
A. phaeospermum (Corda) M.B. Ellis	MAFF 236534	AB220270.1	34	347	90.2
	MAFF 235494	AB220269.1	34	346	90.2
	CBS 463.83	AJ279447	50	503	90.1
	CBS 142.55	AB220256	51	503	89.8
	XSD-91	EU326184.1	35	345	89.8
	XSD-132	EU326200.1	35	345	89.8
	A229	HM222956.1	35	345	89.8
	XSD-08044	FJ478101.1	36	347	89.6
	MAFF 236535	AB220271.1	36	347	89.6
	MUCL 8362	AB220283.1	36	347	89.6
	OUCMBI091005	HQ914944.1	36	347	89.6
	OUCMBI101209	HQ914942.1	36	347	89.6
	IFO 31950	AB220267.1	36	346	89.6
	A218	HM222950.1	36	346	89.6
	A3	AJ279456.1	37	346	89.3
	DSM 62039	AB220261.1	37	345	89.2
A. phaeospermum ?	T57	FJ462766.1	65	521	87.7
A. phaeospermum ?	63/2.4	DQ865110	53	398	86.7
A. puccinioides Kunze	CBS 549.88	AB220253.1	83	347	76.0
A. serenense Larrondo & Calvo	CBS 498.90	AB220240	52	503	89.6
A. sacchari (Speg.) M.B. Ellis	CBS 334.86	AB220257	52	503	89.6
A. saccharicola F. Stevens	ATCC 76288	AB220238	52	503	89.6
Apiospora montagnei Sacc.	CBS 301.49	AB220258	66	521	87.3
Nigrospora sphaerica (Sacc.) E.W. Mason	9038	GQ919077			

* ITS1-5.8S-ITS2 sequence of NFCCI 2144 was subjected to pairwise alignment using Clustal W.

Designated as *Arthrinium phaeospermum* in Genbank

Evolutionary Genetics Analysis (MEGA) software v5.0. (Tamura et al. 2011). The manually edited sequence of NFCCI 2144 was deposited in the NCBI nucleotide sequence database (JF326454) and subjected to a NCBI BLAST search. The partial ITS sequences were aligned using Clustal W together with homologous ITS sequences retrieved from Genbank of the same and related species of *Arthrinium* (Table 1). Due to inconsistent sequence lengths, portions of the ITS1 and ITS2 were excluded from the analysis. To calculate the sequence divergence for ITS, the matrix was analyzed with the Neighbour Joining method (Saitou & Nei 1987) using the Kimura-2 parameter model (Kimura 1980) and Maximum Parsimony method (Tamura et al. 2011). The bootstrap consensus tree (Felsenstein 1985) was inferred from 1000 replicates, with all gaps and missing data eliminated from the dataset (Complete Deletion option).

Taxonomy

Arthrinium rasikravindrii Shiv M. Singh, L.S. Yadav, P.N. Singh, Rahul Sharma & S.K. Singh, **sp. nov.** Figs 1, 2I–P

MycoBank MB 800216

Differs from *Arthrinium phaeospermum* by its larger lenticular conidia and its production of a second elongate type of conidia.

Type: Norway, Svalbard, Spitsbergen, NyÅlesund, 78°55'N 11°56' E, from soil, 27.07.2010, leg. S.M.Singh, (**Holotype,** AMH 9435 [dried colony on PDA]; ex-type culture, NFCCI 2144; GenBank JF326454).

Etymology: The specific epithet refers to Dr Rasik Ravindra, director of the National Centre for Antarctic & Ocean Research and leader of the first Indian Arctic Expedition.

Conidiophores arising from swollen basal cells, micro-semi-macronematous, mononematous, straight or flexuous, unbranched, septate, smooth, thin-walled, hyaline to sub-hyaline, 5–90 × 1–1.5 µm. Conidia arising acropleurogenously, dimorphous i) lenticular, ovoid in face view, 10–15 × 6.0–10.5 µm (n = 50); ii) elongate to clavate conidia, 15–25 × 7.5–10 µm (n = 50), smooth, double-walled, brown to pale olivaceous with prominent truncate base and equatorial germ slit.

Optimum temperature for growth 15–18°C for the arctic holotype (but 25°C for isolates from China and Japan). Colonies on PDA, medium to fast growing, 45–70 mm in 7 days at 18°C (arctic isolate) or 25°C with white, floccose aerial mycelium, reverse uncolored, yellow bright to buff (Rayner 1970). Colony of MAFF410785 slow growing 17.0 mm in 7 days at 25°C, raised dense cottony unusually different from other strains. Colony pattern differing among all four strains tested. Individual strains on OA, PCA, and MEA with similar morphology as on PDA. Sporulation observed after 7 days to 45 days on PCA, as dark pinhead spots in the aerial mycelium near the periphery and later the centre of the colony.

Teleomorph: not observed.

Distribution—China, Japan, Norway.

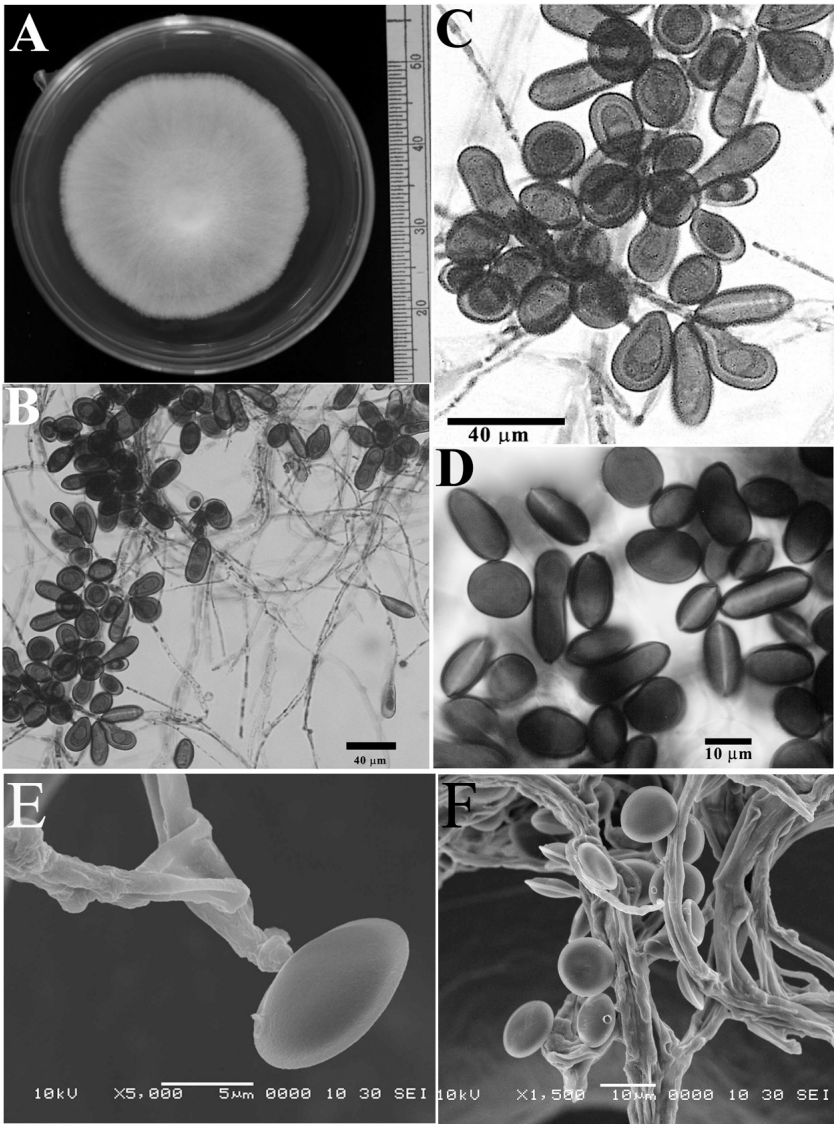


Fig. 1. *Arthrinium rasikravindrii* (NFCCI 2144[T]). A: 7-day old colony on PDA. B–C: Conidia attached to conidiophores. D: Enlarged anomalous and lenticular conidia. E–F: Conidium and conidia under SEM.

Additional material examined—**JAPAN**: Fukushima, isolated from leaf of *Coffea arabica,* T. Sato (MAFF305708); Chiba, isolated from wood tissue of *Pinus thunbergii*, T. Kobayasi (MAFF410785); T. Hasegawa (NBRC6575, IFO6575).

Additional sequenced isolates—**CHINA**: Dalian, isolated from submerged wood collected from marine coast (DLEN2008007); isolated as endophytes from *Oryza granulata* (L10-2-2, L3-4-2). **JAPAN**: Hiroshima, isolated from stubble of *Triticum aestivum*, T. Aoki (MAFF236535, an authentic *A. phaeospermum* strain).

Discussion

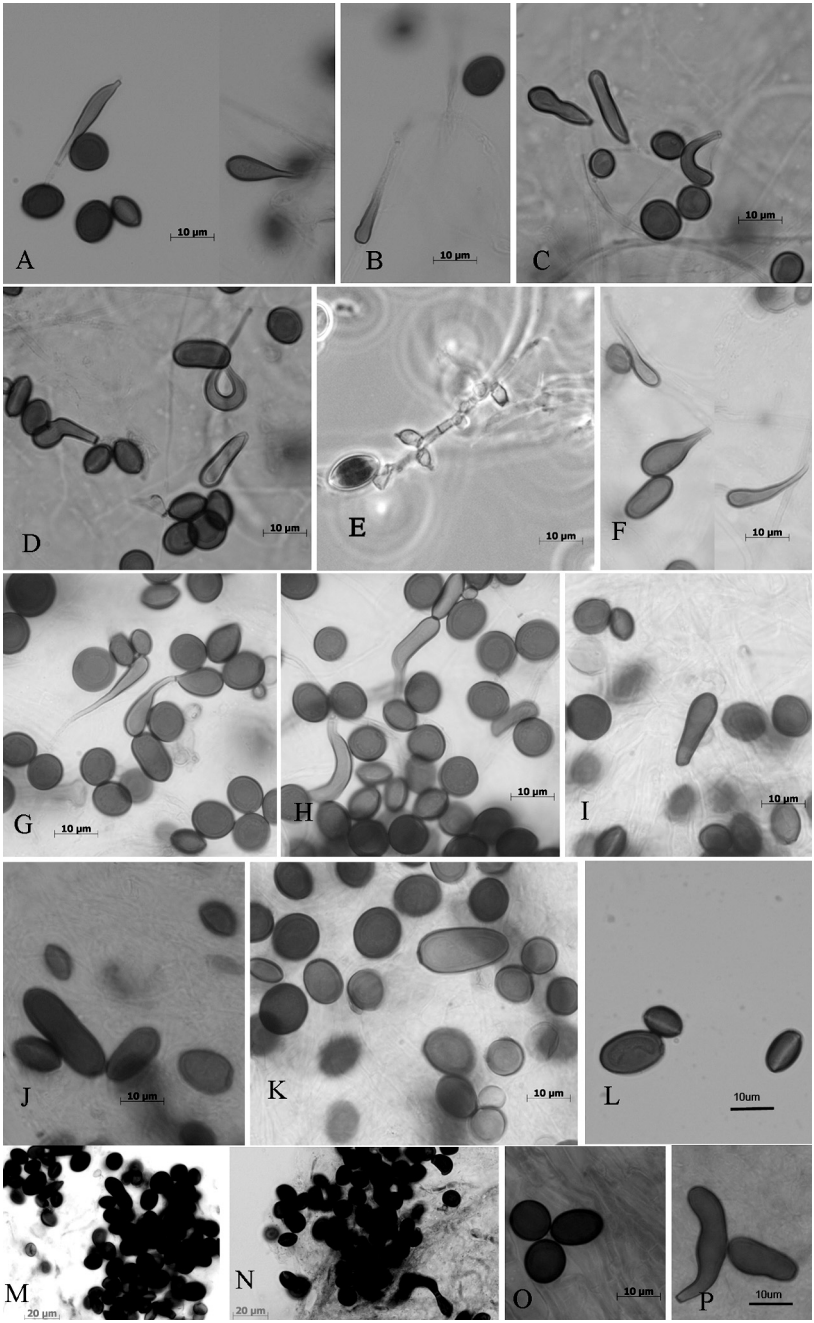
Based on morphological characters and ITS sequence similarity (NCBI BLAST search), the Arctic isolate was determined to belong to *Arthrinium*. Most *Arthrinium* species produce lenticular conidia. As the new species abundantly produces conidia that are lenticular but also elongate, it differs from any previously described *Arthrinium* species, which rarely or never produce elongate conidia.

The NFCCI2144 ITS region comprised a 534 bp nucleotide sequence with 222 ITS1 bp, 150 5.8S bp, and 160 ITS2 bp. A BLASTn search against the Genbank DNA database with this ITS sequence showed maximum similarity (99%) with ambiguous *A. phaeospermum* strains (HM008624.1 query coverage 93%; HM008625.1 query coverage 94%), 97% similarity with *A. aureum* (AB220246.1 query coverage 88%; AB220251.1 query coverage 87%), and only 90% similarity with the isotype strain from *A. phaeospermum* (AB220283.1 query coverage 88%).

A previous study by Khan et al. (2009) of *A. phaeospermum* involving 16 Genbank sequences showed considerable variability among the deposited sequences. Our search of Genbank for *A. phaeospermum* ITS sequences as revealed 25 accessions. A Neighbor Joining tree constructed using these 24 sequences + NFCCI2144 revealed three groups. Our Arctic isolate clustered with six *A. phaeospermum* sequences including IFO6575, MAFF410784, and MAFF305708, which are not monophyletic with the ex-isotype strain of *A. phaeospermum* CBS142.55. Examination of strain IFO6575 revealed the anomalous conidia characteristic of *A. rasikravindrii* and measuring 9.5–22.5 µm × (6.5–)8.5–14.5(–10) µm. Morphological examination of MAFF410785 and MAFF305708 (Fig. 2I–L, O, P) belonging to clade Ia (Fig. 3) and clade IIb (Fig. 4) also showed conidial morphology typical of *A. rasikravindrii*. Their ITS sequences showed a 99.4% and 99.8 % similarity, respectively, to the *A. rasikravindrii* ex-type strain.

Phylogenetic analysis of 10 related species of *Arthrinium* using neighbor-joining method (Fig. 3) placed *Arthrinium* spp. into three clades: clade I contains two subclades — Ia with *A. rasikravindrii* and Ib with two *A. aureum* strains —

Fig. 2. **A–H.** *Arthrinium phaeospermum* (MAFF 236535). A–D, F–H: variously shaped conidia—typical lenticular, globose, and narrow elongated. E: conidiophores. **I–P.** *Arthrinium rasikravindrii*. I–L (MAFF 305708): lenticular, globose, and anomalous conidia. M–N (IFO 6575): lenticular, globose, and anomalous conidia. O–P (MAFF 410784): globose and anomalous conidia.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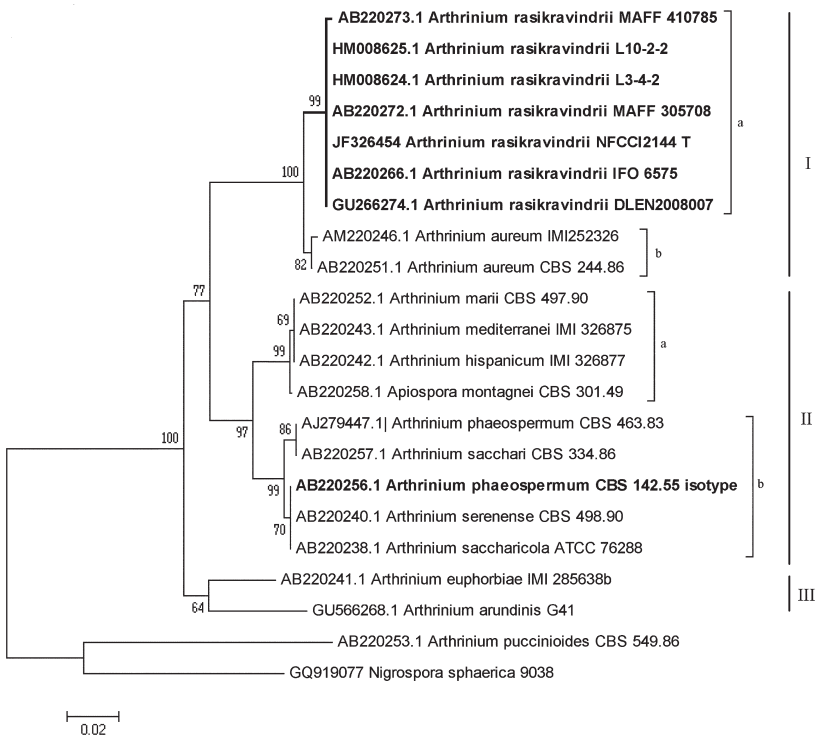

Fig. 3. Neighbor joining tree (1000 bootstrap replications) showing phylogenetic relationship of *Arthrinium rasikravindrii* NFCCI 2144 [T] and 22 ITS sequences deposited in Genbank of authentic strains of 13 *Arthrinium* species. The tree scale (0.02) represents evolutionary distances in units of base substitutions per site as computed by Kimura-2 parameter method. Bootstrap values more than 50 are shown. Gaps are treated as missing data and eliminated from dataset during tree construction. *Nigrospora sphaerica* (GQ919077.1) was taken as outgroup.

clade II includes the most species (8), and clade III comprises *A. euphorbiae* and an *Arthrinium* strain wrongly identified as *A. arundinis* (*Apiospora montagnei*) that is non-monophyletic with the authentic strain of *Apiospora montagnei* CBS 301.49 (clade IIa). Strain IFO6575 and five others are considered to represent *A. rasikravindrii* rather than *A. phaeospermum* as listed in Genbank because they cluster with the holotype isolate and are distant from the isotype strain CBS142.55 of *A. phaeospermum* (Table 1).

A combined analysis of *Arthrinium* spp. (Fig. 4) including all *A. phaeospermum* accessions was consistent with Fig. 3 except that *A. phaeospermum* strains were distributed in three major clusters suggesting fungal sequences incorrectly

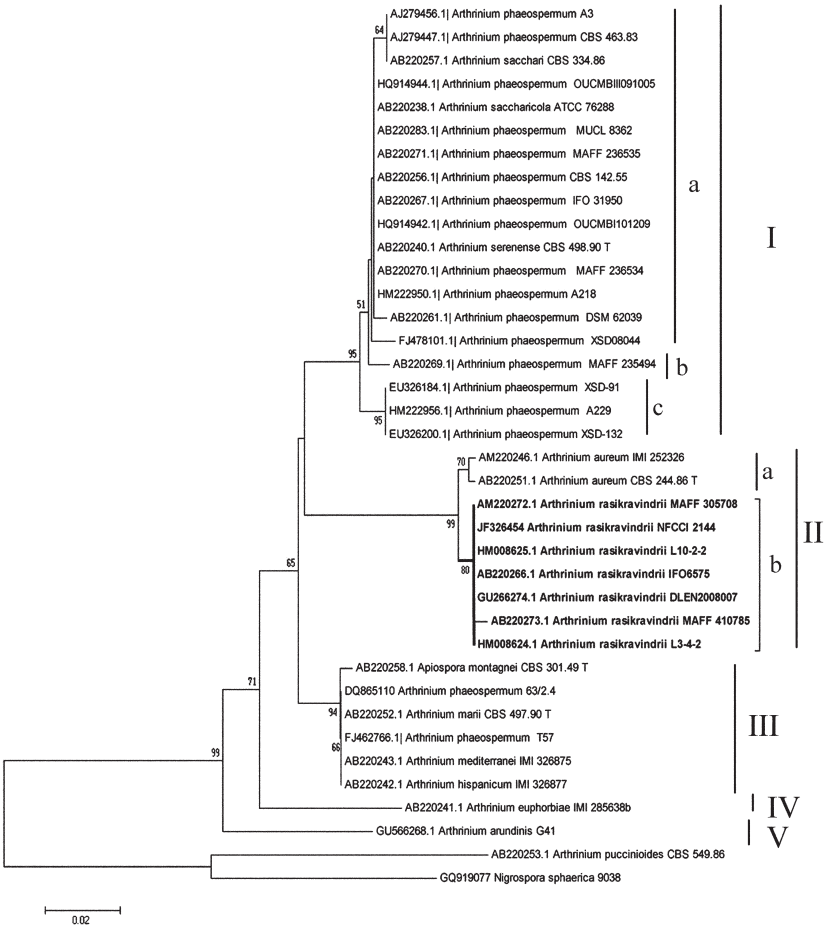

Fig. 4. Neighbor joining phylogenetic tree (1000 bootstrap replications) constructed using ITS sequences of NFCCI 2144^{T} and 24 accessions deposited in Genbank as *Arthrinium phaeospermum* plus accessions of 13 *Arthrinium* spp. The tree scale (0.02) represents evolutionary distances in units of base substitutions per site as computed by Kimura-2 parameter method. Bootstrap values more than 50 are shown. Gaps are treated as missing data and eliminated from dataset during tree construction.

assigned to *A. phaeospermum*. Two strains designated as *A. phaeospermum* belonging to clade III in Fig 4 may also have been misidentified because they are not monophyletic with the *A. phaeospermum* ex-isotype culture CBS142.55 but group instead with *A. hispanicum*, *A. marii*, and *A. mediterranei*. Also three '*A. phaeospermum*' in Fig 4 clade Ic represent an undescribed species.

Arthrinium rasikravindrii differs morphologically from *A. phaeospermum* in having larger conidia and producing a second type of conidia, also seen in an unidentified species of *Arthrinium* by Adelantado et al. (2010) and the *Arthrinium* state of *Apiospora montagnei* by Minter (1985). Additionally *A. phaeospermum* forms narrowly elongated pale brown conidia (Fig. 2A–D, F–H) not seen in any of the examined *A. rasikravindrii* strains (Figs 1, 2I–P). Although *A. phaeospermum* and the *Arthrinium* state of *Apiospora montagnei* form anomalous conidia like *A. rasikravindrii,* these occur only rarely. Genetically, *A. rasikravindrii* differs in 51 positions (10.2%) from *A. phaeospermum* CBS142.55 out of the 503 nucleotides in the ITS region. *Arthrinium rasikravindrii* differs from *A. aureum* having conidia 14–30 × 10–15 µm, and these species differ in the ITS region at 12 nucleotide positions (2.4%). All other species are more than 10% distant from *A. rasikravindrii* in the ITS region. None of the known *Arthrinium* species of form elongate truncate conidia (referred to as anomalous conidia by Adelantado et al. 2010) in abundance except *A. rasikravindrii* (Fig. 1B–D) and the *Arthrinium* state of *Apiospora montagnei* that is genetically (>10%) distant from *A. rasikravindrii* and *A. phaeospermum*.

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